# Differential Induction by Endopolygalacturonase of β-1,3-Glucanases in *Phaseolus vulgaris* Isoline Susceptible and Resistant to *Colletotrichum lindemuthianum* Race β

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**A bioassay allowing absorption of pure endopolygalacturonase (EndoPG) of *Colletotrichum lindemuthianum* race β by near-isogenic lines of *Phaseolus vulgaris* was designed to measure the elicitor effect of the enzyme. β-1,3-Glucanases were retained as defense markers in this system. In response to endoPG, we found that β-1,3-glucanase activity is induced earlier in resistant than in susceptible cultivars to this race of the fungus. Two endo-β-1,3-glucanases that correlated to the appearance of two transcripts of molecular size 2 and 1.6 kb accounted for this increased activity. Elicitation of defense was a time-dependent, saturable phenomenon. It was abolished by the addition of exopolygalacturonase to the bioassay, suggesting that pectic fragments of a critical size are required for endoPG-mediated defense in bean seedlings. The differential ability of an endoPG to elicit near-isogenic lines had not been previously reported.**

*Additional keywords:* gene expression, race-cultivar interaction.

It has been known for a long time that pectin-degrading enzymes are secreted by many plant pathogens when grown *in vitro* in the presence of pectin or plant cell walls. Polygalacturonases and pectin lyases are thought to play an important role during pathogenesis by facilitating tissue colonization through their degradative effect on plant cell walls (English *et al.* 1972).

More recently, it was found that endo-forms of these enzymes may also serve to induce defense responses in plants. They elicit the biosynthesis of phytoalexins and proteinase inhibitors (Bishop *et al.* 1981; Lee and West 1981; Bruce and West 1982; Nothnagel *et al.* 1983; Davis *et al.* 1984) by releasing pectic fragments that act as endogenous elicitors. Elicitation of lignification, of phenylalanine ammonia lyase and β-1,3-glucanases by oligouronides, and by pectic fragments has also been reported (Davis and Halbrock 1987; Robertsen 1987).

The question of the biological significance of pectic endogenous elicitors is currently under investigation in plant-microorganism interactions. Do they act synergistically or independently of microbial elicitors? Do they induce common or independent transduction cascades? Are they released during infection? Most experiments have been performed on plant materials such as cell suspensions or excised tissues (hypocotyls and cotyledons) and almost none of them has thoroughly addressed the question of cultivar specificity. The *Phaseolus vulgaris-Colletotrichum lindemuthianum* race-cultivar specific interaction is well suited for this kind of study. In this system, the extent of pectin degradation by endopolyglacturonase (endoPG) is controlled by the presence of polygalacturonase inhibitory proteins (PGIP) in the host cell wall (Albersheim and Anderson 1971; Lafitte *et al.* 1984). Using gold-labeled PGIP allowed endoPG and endoPG-released fragments to be visualized during infection for the first time (Benhamou *et al.* 1991). This observation reinforced the hypothesis that endoPG plays an important role at the host-parasite interface.

In this work, we study the effect of pure endoPG from *C. lindemuthianum* race β on the elicitation of β-1,3-glucanase in isolines of *P. vulgaris*, susceptible or resistant to this race. It was shown in previous papers that β-1,3-glucanase activity closely reflects the defense status of the host (Kauffmann *et al.* 1987; Kombrink *et al.* 1988; Daugrois *et al.* 1992). Changes in β-1,3-glucanase activity and gene expression in response to endoPG treatment are presented and compared to changes induced upon infection with the same race of the fungus.

## RESULTS

### Endopolygalacturonase.

The enzyme was produced by *C. lindemuthianum* race β grown on synthetic medium containing pectin as the sole carbon source. The purity of the protein, which was isolated according to a previously published procedure (Barthe *et al.* 1981) was checked in this work by SDS-PAGE. Figure 1 shows that the enzyme migrated as a single band, which indicated that it had been purified to apparent electrophoretic homogeneity. A molecular weight of 37,000 was estimated by comparison with standard proteins (Sigma Chemical Co., St. Louis, MO).

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HPLC analysis of the products solubilized by the pure enzyme from polygalacturonic acid (Fig. 2) showed that mono-and digalacturonic acid ($DP_2$) as well as other oligouronides of higher DP had been released, thereby indicating that the enzyme acted as an endopolygalacturonase (English *et al.* 1972; Barthe *et al.* 1981).

**Effect of purified endoPG on β-1,3-glucanase activity in near-isogenic bean lines.**

A time course study of the effect of endoPG was first undertaken on cv. Processor and cv. Earlywax, each cultivar presenting two near-isogenic lines susceptible or resistant to race β of the fungus. Based on preliminary experiments, each cutting was supplied with 2 μg of endoPG (5 nkat). Similar results were obtained whether they were expressed on a fresh-weight basis or on a soluble-protein basis. As shown on Figure 3, absorption of active endoPG led to a marked rise in β-1,3-glucanase activity in the resistant isolines. This phenomenon was not observed after absorption of the heat-denaturated enzyme. Elicitation of β-1,3-glucanase started between 24 and 48 hr in cv. Processor resistant and reached 111% over the control treated with heat-denatured endoPG at 48 hr, and then declined to 66% at 72 hr. In contrast, the effect of endoPG was very low and delayed in the susceptible isoline, only reaching 10 and 20% at 48 and 72 hr, respectively. Since equal amounts of enzyme solution were absorbed during the same time, this differential effect was not due to a different rate of endoPG absorption. The responses of susceptible and resistant isolines of cv. Earlywax to race β were in the same order of magnitude, 8 and 174%, respectively, at 48 hr. Again, an earlier and higher induction of β-1,3-glucanase was recorded in resistant than in susceptible plants. β-1,3-Glucanase activity evolved similarly in the controls, whatever susceptible or resistant, after absorption of denaturated endoPG. A slight increase, which tended to become greater after 3 days, was recorded. This probably reflected aging of the cuttings; therefore, the experiments did not exceed 3 days. Cultivar Processor was retained for the following experiments.

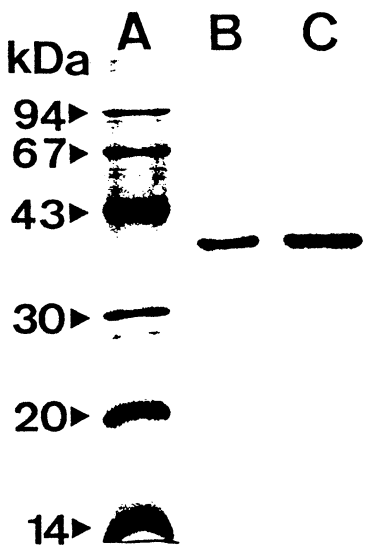


**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of endopolygalacturonase purified from the culture filtrate of *Collectotrichum lindemuthinum* race β: Molecular weight markers (lane A), endoPG 1.5 μg (lane B), and 3 μg (lane C).

**Elicitation in different organs.**

The cuttings were supplied with endoPG, and then with water for an additional 48 or 72 hr, before being dissected into hypocotyls and leaves. The cotyledons were discarded because of the presence in crude extracts of material interfering with the measurement of β-1,3-glucanase activity. As shown in Figure 4, the elicitor effect of endoPG was much more pronounced in leaves than in hypocotyls, both at 48 and 72 hr. Although *C. lindemuthianum* is a pathogen of both stems and leaves, this difference probably reflects the fact that, as in other plants, PR proteins such as β-1,3-glucanases and chitinases are typically induced in leaf tissues. Besides being organ specific, the response was again higher in the resistant than in the susceptible isoline.

It was described in a previous paper that, upon infection, resistant bean seedlings showed a two- to threefold increase in β-1,3-glucanase activity and that this increase occurred earlier than in their near-isogenic susceptible counterparts (Daugrois *et al.* 1990). From the data reported in Figures 3 and 4, it appears that the two- to threefold elicitation of β-1,3-glucanase by endoPG closely mimics the differential induction of this enzyme during infection of intact plants by race β of the fungus. We did not look for the effect on cv. Processor resistant of an endoPG from a compatible race, for instance race K58, because it was previously found that, in contrast to race β, infection by this race did not lead to early induction of β-1,3-glucanases (Daugrois *et al.* 1990).

**Dose-response effect.**

The cuttings were supplied with increasing amounts of endoPG ranging from 0.4 to 6 μg (1 to 7.5 nkat). After 48 hr it was found that the elicitation effect was a dose-dependent phenomenon; at 2 μg of endoPG in the medium β-1,3-glucanase activity was more than twofold increased (Fig. 5), while higher amounts lowered the response. The elicitation process was totally abolished when the exopoly-

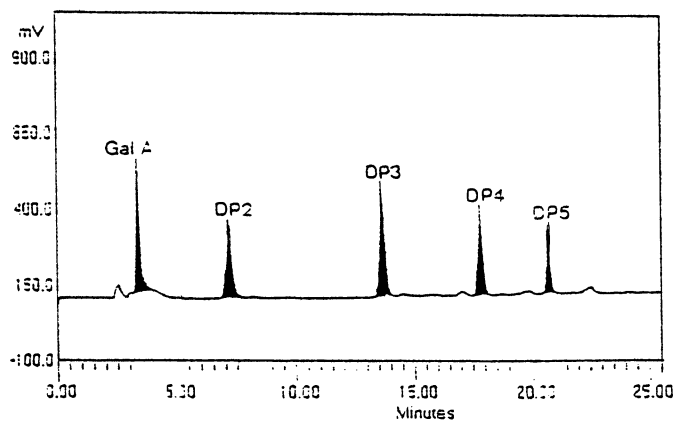


**Fig. 2.** Anion exchange high-performance liquid chromatography of the products released from polygalacturonic acid by the pure endoPG of *Colletotrichum lindemuthianum* race β. Mono- and digalacturonic acid ($DP_2$) as well as oligouronides of higher DP were obtained.

galacturonase (4 μg = 5 nkat) also purified from the same fungus (Barthe *et al.* 1981) was supplied together with endopolygalacturonase (2 μg = 5 nkat).

**Partial characterization of endoPG-induced β-1,3-glucanases.**

Two days after absorption of endoPG, protein extracts were prepared from the primary leaves of resistant and susceptible seedlings and from the corresponding controls. Extracts prepared from 3-day-old infected seedlings were retained for comparison. Ion-exchange chromatography of the extracts on CM-Sephadex C50 showed two peaks of constitutive β-1,3-glucanase activity in the near-isogenic control leaves (Fig. 6A). One peak was not retained, whereas the other one was eluted at 0.6 M NaCl in the gradient. Two additional peaks were recovered from the extracts of endoPG-elicited resistant leaves (Fig. 6B) and from race β-infected resistant seedlings (Fig. 6C). They were barely detectable in extracts from susceptible plants at the same stage, whatever treated by endoPG or infected and were similarly eluted at 0.35 and 0.45 M NaCl. Again elicitation by endoPG race β discriminates the near-isogenic lines, with the same specificity as the live race β fungus itself. It had been shown elsewhere that the two activities induced upon infection corresponded to two basic endo-β-1,3-glucanases of MW 36,500 and 36,000 called GE1 and GE2. Looking at the high level of constitutive β-1,3-glucanase activity, it is quite clear that expressing the data over the control does not reflect the high induction of each specific form. Moreover, the presence of stem tissues in the extracted plant material led to a dilution of the induction fold, since β-1,3-glucanase activity was not increased in these organs upon elicitation.

Their endolytic mode of cleavage was checked by HPLC analysis of the products obtained from laminarin. The two enzymes induced by endoPG or infection released a series of oligoglucosides of DP ranging from 2 to 6 or more (data not shown), thereby indicating that they acted as endoenzymes.

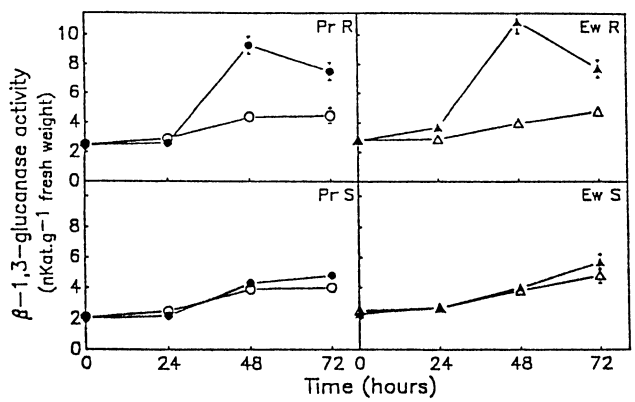


**Fig. 3.** Time course measurement of β-1,3-glucanase activity in cuttings of cv. Processor (Pr, ●–●) and cv. Earlywax (Ew, ▲–▲) susceptible (S) or resistant (R) to *C. lindemuthianum* race β after absorption of the endopolygalacturonase isolated from the culture filtrate of the fungus. Each cutting received 2 μg of pure endoPG. Heat-denaturated endoPG was used in the controls ( ○–○, △–△). Each value represents the mean of three experiments ± SE to the mean; for most values standard deviation was within the size of the symbols.

**β-1,3-Glucanase gene expression.**

To gain insight into the elicitation process, we made a kinetic study of β-1,3-glucanase gene expression in cv. Processor resistant to race β, in response to endoPG or infection. Northern blot experiments (Fig. 7) showed that two transcripts of approximately 2 and 1.6 kb, and probably an intermediary species, were increased in the two situations. These increases started 12 hr after elicitation and 72 hr after inoculation, and lasted for 56 hr and 6 days, respectively. The kinetics of their increases and their intensity are in agreement with the timing of induction upon infection or elicitation of the two new β-1,3-glucanases shown on Figure 6 (Daugrois *et al.* 1990, Daugrois *et al.* 1992). The smaller species might correspond to the transcript induced by fungal elicitor in bean cell suspension cultures (Edington *et al.* 1991). Some hybridization signals were occasionally seen in control samples that might reflect either a wounding effect or/and expression of constitutive β-1,3-glucanase genes; these possible effects were not followed by corresponding increases in enzymic activity. They might also result from the mild stringency conditions that were retained, attributable to the heterologous probe which was used. From the overall data, we concluded that endoPG stimulates β-1,3-glucanase activity by affecting gene expression.

## DISCUSSION

In a previous work, we showed that *C. lindemuthianum* produces large amounts of endoPG upon infection of its host (Benhamou *et al.* 1991). The present study was under-

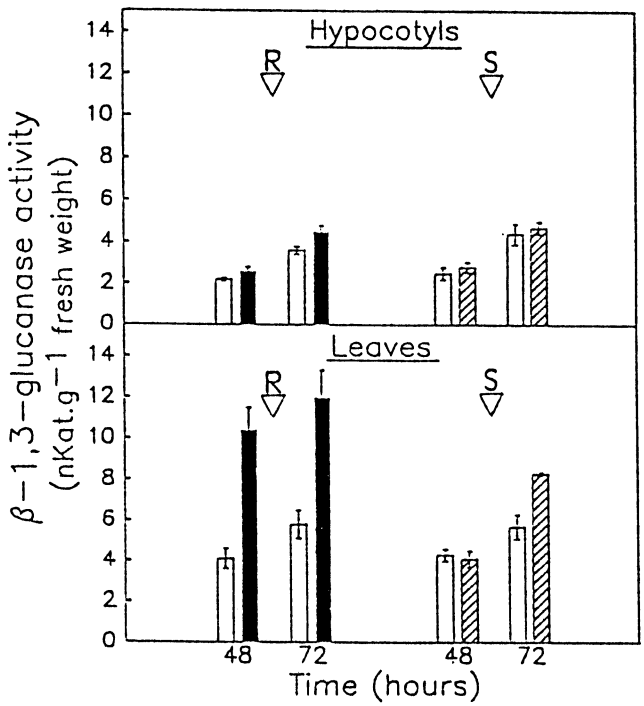


**Fig. 4.** β-1,3-Glucanase activity in the leaves and hypocotyls of cv. Processor resistant (solid bar) or susceptible (striped bar) to *Collectotrichum lindemuthianum* race β, 48 and 72 hr after absorption of pure endoPG (2 μg per cutting). Heat denatured endoPG was used in the controls (open bar). The data represent the mean of three experiments ± SE to the mean.

taken to assess the contribution of endoPG to signaling phenomena leading to defense. A bioassay allowing absorption of the pure enzyme isolated from *C. lindemuthianum* race β was first designed. Cuttings consisting in the shoot portion of bean seedlings susceptible or resistant to race β were used for this purpose, and β-1,3-glucanase was retained as a defense marker. The obtained data indicated that endopolygalacturonase of race β shows a differential ability to elicit endo-β-1,3-glucanases in near-isogenic lines of the host resistant or susceptible to this race. Amounts as low as 27 pmoles (1 μg) of the enzyme were sufficient to induce this response. Elicitation of defense occurred early in resistant seedlings and only in a delayed manner in susceptible ones. Thus, endoPG mimics the effect of the fungus itself on defense induction (Daugrois *et al.* 1990) in that it discriminates susceptible and resistant seedlings. Similar stimulation levels, two- to threefold above the control values, were observed during infection and elicitation. This induction increase reflected the presence of constitutively expressed β-1,3-glucanases and resulted from the appearence of two new enzyme forms which were, in themselves, induced to a much higher level.

Elicitation of endo-β-1,3-glucanase by endoPG was saturable and time dependent and required β-1,3-glucanase gene expression. The saturability of the phenomenon suggested an enzyme-substrate mode of interaction. The fact that exopolygalacturonase abolished the elicitation response further indicates that pectic fragments of a critical size are required for endoPG-mediated defense in bean seedlings. *In planta*, the presence of PGIP might prevent a too extended degradation through its interaction with endoPG, hence controlling the level of endogenous signaling, as proposed by Cervone *et al.* (1989). Indeed, the previously reported higher amounts of constitutive PGIP in resistant than in susceptible seedlings (Lafitte *et al.* 1984) might explain the differential effect recorded in this work. Alternatively, or additionally, the possibility that the two isogenic lines contain slightly different pectic polysaccharides giving rise to different fragments, cannot be ruled out.

The systemic induction of defense in this system also addresses the question of signal migration. Possible candidates are the endoPG itself, the pectic elicitors it releases, or other elicitor-generated signals. The recently reported effect of pectic fragments on membrane potential (Mathieu *et al.* 1991) leading to membrane depolarization, might account for electric systemic signals. The local and long-distance effects of such signals are not known.

Pectic enzymes are supposed to play important roles both in pathogenesis and in signaling phenomena (Albersheim *et al.* 1969; Bateman and Basham 1976; Collmer and Keen 1986). This study is the first demonstration that the endoPG of *C. lindemuthianum* race β elicits a defense response in its host and that this elicitation mimics the differential effect of infection by this race on near-isogenic lines of the host. This does not imply that specificity in this system is primarily determined by endoPG. Indeed, the endopolygalacturonases from three different races of *C. lindemuthianum* appeared very similar in terms of their amino acid sequences (Keon *et al.* 1990). It may be hypothesized, however, that endoPG and PGIP enter some step of the recognition cascade. The differential effect resulting from this interaction would then be amplified by early release of elicitorlike, β-1,3-glucan-containing fragments from the fungus in the incompatible interaction,

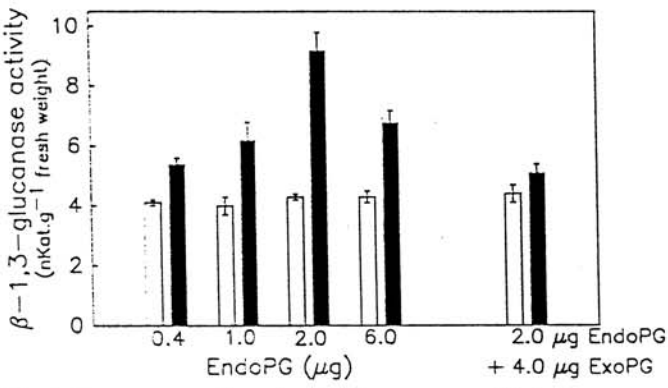


**Fig. 5.** Dose response effect of endoPG treatment on β-1,3-glucanase elicitation in cv. Processor bean cuttings resistant to *C. lindemuthianum* race β, 48 hr after absorption of the enzyme (solid bar). Heat-denatured endoPG was used in the controls (open bar). Each value represents the mean of three experiments ± SE to the mean.

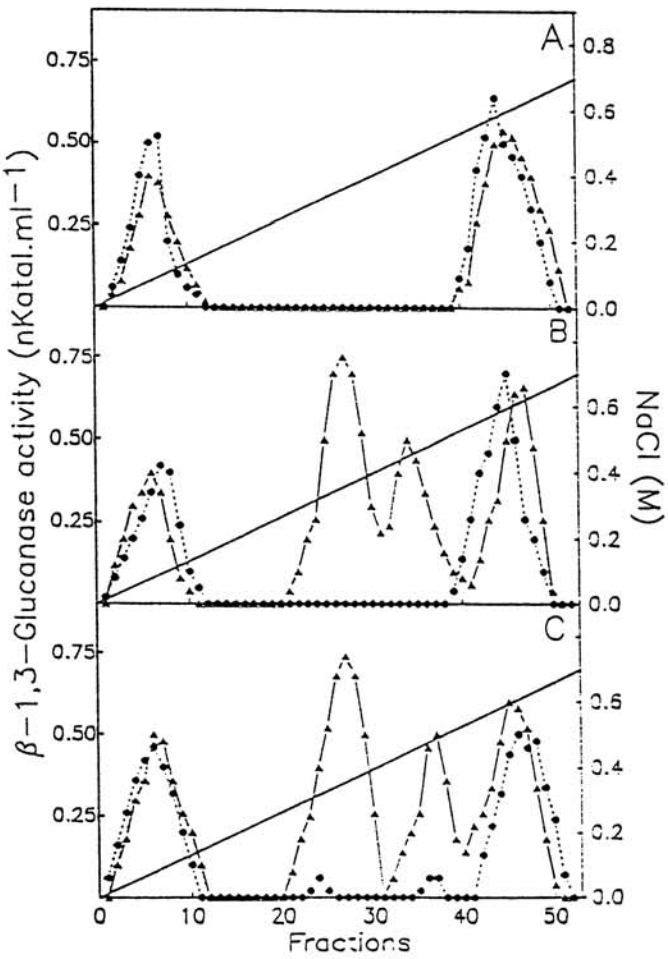


**Fig. 6.** Chromatography on a CM-Sephadex C50 column of β-1,3-glucanases present in extracts from cv. Processor resistant (Δ–Δ) or susceptible (●–●) to race β of *C. lindemuthianum*: **A,** control seedlings; **B,** 48 hr after absorption of pure endoPG; **C,** 72 hr after inoculation by the fungus.

via the action of the two newly induced endo-β-1,3-glucanases. Additional experiments are needed to check this hypothesis. The effect of endoPG from race K58 which is compatible with the near-isogenic lines used in this work should be investigated. Further experiments will be aimed at understanding whether the enzymes from races β and K58 mimic the race-cultivar specific induction of β-1,3-glucanases which was recorded from infected seedlings.

The role played by endopolygalacturonase in fungal pathogenicity was questioned in a recent work by Scott-Graig *et al.* (1990) who showed that endoPG gene disruption did not affect the ability of *Cochliobolus heterostrophus* to invade corn seedlings. Although such a critical experiment has not been reported yet in *C. lindemuthianum*, the marked degradation of pectic polysaccharides in infected bean tissues suggests that, besides being involved in signaling phenomena, this enzyme is also required for fungal pathogenicity in this system.

## MATERIALS AND METHODS

### Biological material.

Near-isogenic lines of *P. vulgaris* 'Processor' and 'Earlywax', susceptible or resistant to race β of *C. lindemuthianum* (Bannerot 1965) were retained in this study. The seedlings were grown in a greenhouse as described (Lafitte *et al.* 1984). The fungus, *C. lindemuthianum* race β was maintained on glucose-peptone agar. The conidia obtained from a 6-day-old culture of the fungus were suspended in sterile water and used for plant inoculation as described previously (Lafitte *et al.* 1984).

### Endopolygalacturonase.

Homogeneous α-1,4-D-endopolygalacturonase (E.C. 3.2.1.15) was prepared from a culture of *C. lindemuthianum* race β according to the procedure described earlier by Barthe *et al.* (1981). EndoPG activity was measured spectrophotometrically by following the release of reducing groups (Somogyi 1952) from 0.1% polygalacturonic acid (NBC) in acetate buffer 0.2 M pH 5.2 at 30° C for 30 min. One nanokatal corresponds to the release of 1 nmol galacturonic acid equivalent per second in the conditions of the assay (total volume was 1 ml). The purity of the enzyme preparation was checked by SDS-PAGE analysis according to Laemmli (1970). The gels were stained with silver nitrate according to Morrissey (1981).

### Elicitor bioassay.

Seven-day-old bean seedlings which were at the first primary leaf stage were cut off 2 cm below the cotyledons. Each cutting was placed in a small vial and allowed to absorb 0.3 ml of an endoPG solution in water containing 0.1 mg·ml$^{-1}$ streptomycin (Sigma) through the cut section of the hypocotyl. Absorption of the elicitor was completed after 4–6 hr at 22° C in a growth chamber under light. The cuttings were then allowed to absorb water plus streptomycin (0.1 mg·ml$^{-1}$) for an additional 4–48 hr under cycles of 14 hr light at 22° C and 10 hr dark at 18° C. Controls were supplied with heat-denatured enzyme in the same conditions.

### β-1,3-Glucanase assay.

Total β-1,3-glucanase activity was determined by measuring the amount of reducing sugars released from

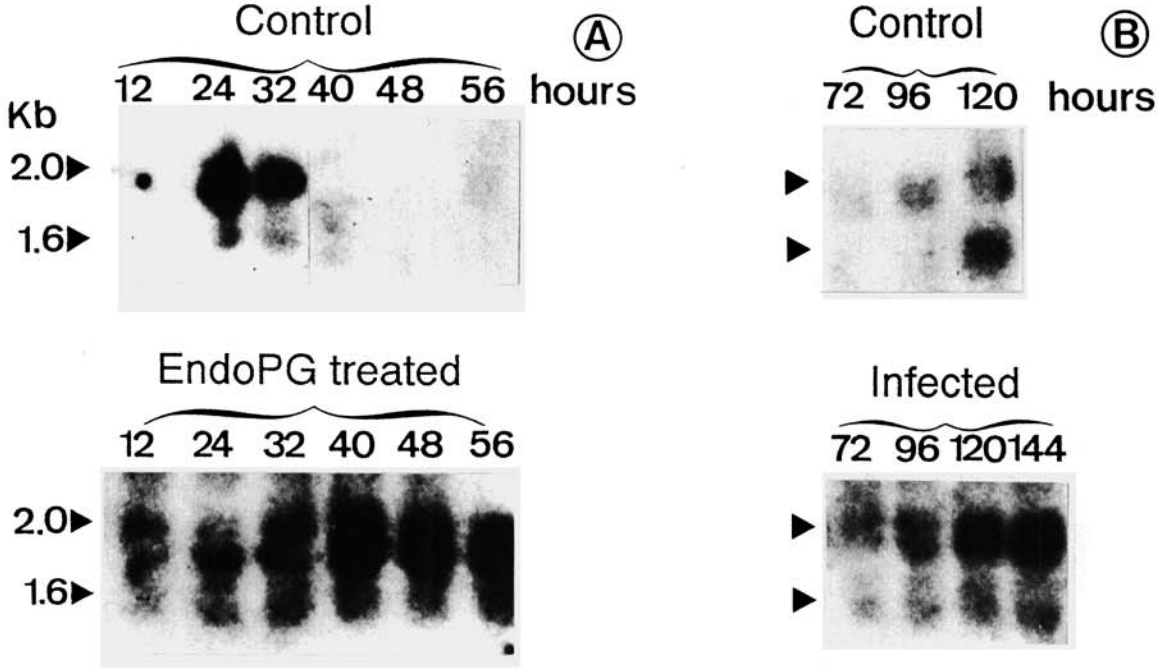


**Fig. 7.** Time course of the accumulation of β-1,3-glucanase transcripts in: **A,** Cuttings of cv. Processor resistant to *Colletotrichum lindemuthianum* race β after absorption of the endopolygalacturonase (2 μg per cutting) isolated from the culture filtrate of the fungus; **B,** Seedlings of the same cultivar after inoculation with the same race. The controls were treated with heat-denaturated endoPG (A) or sprayed with water (B). Northern hybridization was carried out with a tobacco β-1,3-glucanase partial cDNA probe.

laminarin (Calbiochem). The enzyme extract was obtained from bean cuttings (4 g) ground at 4° C for 5 min in 50 ml of acetate buffer 50 mM, pH 5.2, 1 M NaCl, 1% Polyclar AT. The suspension was stirred for 20 min at 4° C, filtered through a coarse glass filter and the filtrate centrifuged at 1,500 *g* for 10 min. The supernatant was dialyzed for 4 hr against 5 L of acetate buffer 20 mM, pH 5.2. Total β-1,3-glucanase activity was measured on this dialyzed crude extract. The assay (1 ml) contained enzyme extract, 0.1 M acetate buffer, pH 5.2, and 2.5 mg of laminarin which was previously dissolved in the same buffer by heating at 60° C before use. After 30 min of incubation at 50° C, the reducing sugar content was determined according to the method of Somogyi (1952) and catalytic activity was expressed in nanokatals.

**β-1,3-Glucanase chromatography.**

Enzyme extract obtained from bean leaves (5 g) as described earlier (Daugrois *et al.* 1992) was dialyzed against 25 mM acetate buffer (pH 5.2) and applied to a CM-Sephadex C50 column (20 × 1.6 cm) equilibrated in the same buffer. Elution was performed with a linear gradient of NaCl (0–0.7 M); fractions (4 ml) were collected at a flow rate of 40 ml/hr and analyzed for their β-1,3-glucanase activity.

**RNA isolation and gel blot hydridization.**

Total RNA was extracted from bean cuttings as described by Haffner *et al.* (1978), denaturated with formamide/formaldehyde and submitted to electrophoresis in a 1.2% (w/v) agarose gel containing formaldehyde. Equal loading of the lanes (10 μg of total RNA) was checked by ethidium bromide staining. Gels were blotted onto nitrocellulose filters. The filters were air-dried, baked at 80° C in a vacuum oven, and prehybridized (4 hr) at 42° C in 50% formamide, 2× SSC, 2× Denhardt's solution, 0.1% SDS, and 0.1 mg/ml denatured calf thymus DNA. Hybridization to a β-1,3-glucanase probe, corresponding to the insert of a partial tobacco cDNA clone kindly provided by Y. Marco (Godiard *et al.* 1991), was carried out for 24 hr in the same conditions. The probe (15 ng/ml) was labeled by random priming with [$\alpha^{32}$]dNTPs. Filters were then washed in 2× SSC, 0.1% SDS first at room temperature and then at 55° C until the background was acceptable for autoradiography. One brief final wash in 2× SSC at 55° C was then performed before exposure.

**Protein measurement.**

Protein were detected in column eluates by measuring the absorption at 280 nm. The concentration of endopolygalacturonase was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

**Sugar analysis.**

The products obtained by enzymic digestion of polygalacturonic acid and laminarin were analyzed by anion-exchange chromatography with a Dionex (Sunnyvale) Bio-L HPLC system equipped with a CarboPac PA1 column (4 × 250 mm) according to Hotchkiss and Hicks (1990).

## ACKNOWLEDGMENTS

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